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The Sequencing, Synthesis, and Biological Actions of an ANP-Like Peptide Isolated from the Brain of the Killifish *Fundulus heteroclitus*

D. A. PRICE[1], K. E. DOBLE[1], T. D. LEE[2], S. M. GALLI[3], B. M. DUNN[4], B. PARTEN[4], AND D. H. EVANS[5]

[1]*The Whitney Laboratory, St. Augustine, Florida 32086,* [2]*Division of Immunology, Beckman Research Institute of the City of Hope, Duarte, California 91010,* [3]*Department of Physiology, School of Medicine, University of Florida, Gainesville, Florida 32611,* [4]*Department of Biochemistry, School of Medicine, University of Florida, Gainesville, Florida 32611, and* [5]*Department of Zoology, University of Florida, Gainesville, Florida 32611*

Abstract. We have extracted, purified, and sequenced an ANP-like peptide from the killifish. The peptide was extracted from whole brains with acidic acetone, and the aqueous phase remaining after evaporation of the acetone was subjected directly to HPLC. A pure peak was obtained after three successive HPLC steps. A key part of our purification method was the deliberate oxidation of methionyl residues in the peptide between the second and third HPLC steps. The purified peptide was chemically sequenced, and its molecular weight was determined by fast atom bombardment mass spectrometry (FABms). The peptide is 22 amino acids long and has considerable sequence similarity to the known natriuretic peptides, especially within the disulfide bonded "ring"; but unlike these known peptides it ends immediately after the second half cystine. Though it lacks a C-terminal "tail," the killifish peptide is equipotent to rat ANP in our radioimmunoassay, which employs an antiserum to the rat peptide. Furthermore, this brain peptide is equipotent to eel ANP in relaxing toadfish aortic rings, though both fish peptides are slightly less potent than rat ANP.

Introduction

The natriuretic and diuretic activities of mammalian atrial extracts were first reported in 1981 (DeBold *et al.*, 1981) and were soon ascribed to various atrial natriuretic peptides (ANPs) ranging in molecular weight from 2 kD to about 13 kD. In the past nine years, natriuretic peptides have also been isolated from brain, and the genetic basis of the family has been clarified.

Each mammalian species has two genes encoding ANP-like peptides: an A gene and a B gene. The precursor arising from the A gene is gamma-ANP; it contains 126 amino acid residues (13 kD), and corresponds to the primary gene product minus the signal sequence (Kangawa *et al.*, 1984). Similarly, the B gene gives rise to a precursor designated gamma-BNP (Sudoh *et al.*, 1988, 1989; Kojima *et al.*, 1989; Seilhamer *et al.*, 1989).

Both the A and B genes are expressed in the hearts of all of the mammalian species examined so far, and ANP is present in all of the brains. However, the expression of the B gene in the brain, and the relative abundance of its products in heart and brain, seem to vary with species. In the pig, for example, the B precursor is somewhat (a few fold) more prevalent than the A in brain, whereas the A form is much more common (50 times) than the B in atrium (Aburaya *et al.*, 1989b). In contrast, the B precursor in the rat is not expressed at all in brain and, in fact, accounts for only a minor portion of the activity (compared to ANP) in any tissue (Aburaya *et al.*, 1989a).

The most abundant product of the A gene in atria is gamma-ANP, itself (126 residues), but active peptides as short as 21 amino acids have also been isolated from rat atria (reviewed by Lewicki *et al.*, 1986). All of the smaller peptides corresponded to fragments of gamma-ANP.

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Abbreviations: Atrial Natriuretic Peptide (ANP), Brain Natriuretic Peptide (BNP), Fast Atom Bombardment mass spectroscopy (FABms).

The precursors derived from the B gene in various species have amino acid differences that affect their post-translational processing. In rat atrium, for example, the B gene gives rise to a 45 amino acid peptide and no smaller products have been found (Flynn *et al.*, 1989). However, in the pig brain, gamma-BNP is processed to both 32 and 26 amino acid forms (Sudoh *et al.*, 1988), but these smaller peptides seem not to occur in human brain (Sudoh *et al.*, 1989).

Regardless of size or tissue of origin, all of the biologically active peptides contain a disulfide-linked ring of 17 amino acid residues. This ring is essential for ANP-like biological activity (Misono *et al.*, 1984).

ANPs have also been isolated and sequenced from the atria of sub-mammalian vertebrates (Miyata *et al.*, 1988; Sakata *et al.*, 1988; Takei *et al.*, 1989; sequences are shown in Fig. 5), and ANP-like immunoreactivity occurs in the brain as well. In the toadfish, the total brain levels of ANP-like immunoreactivity are comparable to those of the heart (Galli *et al.*, 1988); in contrast, mammalian heart expresses much higher levels of both ANP and BNP precursors than the brain (Sudoh *et al.*, 1989). This may reflect a relatively greater physiological role for brain ANP in fish compared to mammals, so we thought it important to isolate and sequence an ANP-like peptide from fish brain.

Materials and Methods

Radioimmunoassay (RIA)

We used an antiserum to rat ANP provided by M. I. Phillips (University of Florida), together with iodinated synthetic rat ANP (Peninsula Laboratories), to provide an RIA. Rat ANP was iodinated with chloramine T and separated from unbound iodide on a C18 Sep-Pak (Waters); the methods were basically the same as those used previously with FMRFamide (Price, 1982). Two modifications are important, however: first, contact with metabisulfite must be minimized (5–10 s); second, the peptide should be eluted from the Sep-Pak with 80% aqueous acetonitrile containing 0.1% trifluoroacetic acid, rather than methanol. In some cases, we purified the iodinated peptide by HPLC, but this could be avoided if the metabisulfite contact was sufficiently brief. In the actual assay, we also used the same buffers and charcoal suspension as for the FMRFamide RIA.

When the sequence of eel ANP was determined, some synthetic peptide became available to us (a kind gift from Y. Takei and the Protein Research Foundation of Japan). We have tested it in the RIA with rat ANP trace and have also iodinated it (as above) and used it as trace.

Extraction and purification

Fundulus brains were kindly provided (over the course of weeks) by Peter Lin (Whitney Laboratory) who was dissecting the fish in order to collect the pituitaries. We accumulated the brains (30–40 mg each) in flasks containing acidic (0.1% trifluoroacetic acid) acetone to a final concentration of 2 brains/ml. The flasks were kept at −20°C between successive additions.

When sufficient material had been collected, the acetone was decanted from the brains and clarified by centrifugation. The acetone was removed on a rotary evaporator, leaving an aqueous fraction which was centrifuged, filtered, and neutralized to pH 7.0 with dilute sodium hydroxide solution. The solution was pumped onto an HPLC column (Aquapore Octyl Prep 10, 100 × 10 mm, from Brownlee) at 4 ml/min. After loading, the column was washed with 0.1% aqueous TFA, and then with 16% acetonitrile in 0.1% TFA. Finally, a gradient of acetonitrile (16%–40% over 30 min) was started. Half-minute fractions were collected, and 2 μl aliquots were taken from each fraction for RIA.

The immunoreactive fractions were pooled, diluted with water containing 0.1% TFA (2–3 times the original volume), and applied to an RP-300 (Brownlee) column (220 × 2.1 mm) by pumping at .5 ml/min. Elution of the column was performed with the same gradient as described for the Prep 10 above. The major immunoreactive fraction was oxidized with 1.5% hydrogen peroxide for 15 min and re-run on this same HPLC system.

Sequencing and FAB mass spectrometry (FABms)

The peak immunoreactive fraction from the third HPLC step was divided in half. The half to be used for FABms analysis was dried on a Speed-Vac, and mailed to the mass spectrometry laboratory. There, the residue was redissolved in a very small (few μl) volume of dimethylsulfoxide, and 1–2 μl of this was used for analysis as described previously (Bullock *et al.*, 1988). The remaining half (about 0.1 ml) of the fraction was applied (in 3 portions with intermediate drying) directly to a preconditioned glass-fiber filter disk containing 3 mg of Polybrene. The disk was placed in the sequencer (Applied Biosystems 470A gas-phase sequencer with an on-line 120A PTH analyzer), and the PTH-amino acid derivatives in each cycle were identified by their retention times and quantitated by comparison of the peak areas to standards.

Synthesis

The peptide G-W-N-R-G-C-F-G-L-K-L-D-R-I-G-S-M-S-G-L-G-C was synthesized on an Applied Biosystems 430A peptide synthesizer starting from t-Boc-Cys(4-CH_3-benzyl)-PAM resin (Applied Biosystems) on a 0.1 mmole scale. Each amino acid was added in a double coupling procedure using the manufacturer's programs. The complete resin was recovered in 77% yield

Table I

Amino acid compositions of synthetic ANP

Amino acid	Ratios		
	Crude found	Pure found	Theor.
Glycine	5.93	5.76	6
Leucine	3.14	3.25	3
Arginine	1.90	2.13	2
Methionine	.60	1.02	1
Phenylalanine	.93	.85	1
Serine	1.95	1.97	2
Aspartic acid	2.07	2.13	2
Isoleucine	.99	1.00	1
Lysine	1.05	.83	1
Cystine (1/2)	+	1.98	2

+: Present, but not quantified.

by weight. The peptide was removed from the resin with anhydrous HF/anisole (9/1) at 0°C for 1 h. The peptide was extracted with 10% acetic acid/water and yielded 118 mg after lyophilization. The amino acid analysis is shown in Table I (crude). The formyl group (from the indole ring of Trp) was removed and the disulfide bond formed by bubbling air through a 5×10^{-5} *M* solution of peptide in .5 *M* ammonium bicarbonate, which had been adjusted to pH 8.7 with ammonium hydroxide. The peptide was purified by HPLC and quantitated by amino acid analysis (Table I, pure).

Vasorelaxant activity

Rings cut from the ventral aorta of toadfish (*Opsanus beta*) were suspended in physiological saline and their tension recorded. Peptide solutions were added and the drop in tension recorded (Evans *et al.*, 1989).

Results

Only one major peak of immunoreactivity showed up on the first HPLC fractionation (Fig. 1), but often there were two peaks at the second step separated by about 2 min in elution time (Fig. 2A). We suspected that the earlier-eluting peak might be an oxidation product of the second, and confirmed this by oxidizing the later peak, thereby shifting its retention time to that of the earlier peak (Fig. 2B). This shift is sufficient to move the ANP-like peak out from under the impurities remaining at the second step. We judged the peak to be pure enough for sequencing after the third step. Thus, deliberate oxidation allows purification of the peptide in fewer steps than would otherwise be required.

From about 1500 brains (50 g total) we isolated a peak of immunoreactivity from which we were able to identify 20 of the first 21 amino acid residues from the peptide by sequencing (Fig. 3). At position 6, only dehydro-alanine was present, which is suggestive of the half cystine we would expect by comparison to other ANP-related peptides. In cycle 22, where we expected the other half cystine, no clear signal was observed, nor were any detectable thereafter.

FAB mass spectral analysis showed a prominent protonated molecular ion at m/z 2341.2 (Fig. 4B), which was in good agreement with the value of 2341.1 calculated for the cyclic peptide with a methionine sulfoxide and ending with the second half cystine. The doubly protonated molecular ion was observed at m/z 1171. The ion distribution of the protonated molecular ion cluster was in substantial agreement with that calculated from

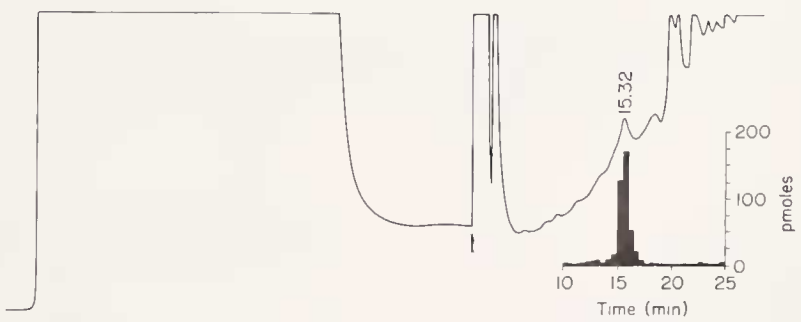

Figure 1. HPLC fractionation of the aqueous fraction remaining after evaporation of acetone from the initial extract (Brownlee Aquapore Octyl column, 100 × 10 mm). The UV absorbance at 210 nm (0.4 AU full scale) is plotted against time, and the immunoreactivity is shown as a histogram. The solvent is pumped at 4 ml/min throughout, and fractions of 0.5 min are collected during the gradient elution. The sample is loaded through the pump inlet and washed in with aqueous HPLC buffer (0.1% TFA); the absorbance remains off-scale during the loading, but drops down during the washing. When the absorbance has leveled off, the flow is switched to 16% ACN in 0.1% TFA, and when this solvent reaches the detector (arrow), a linear gradient is started to 40% ACN at 30 min.

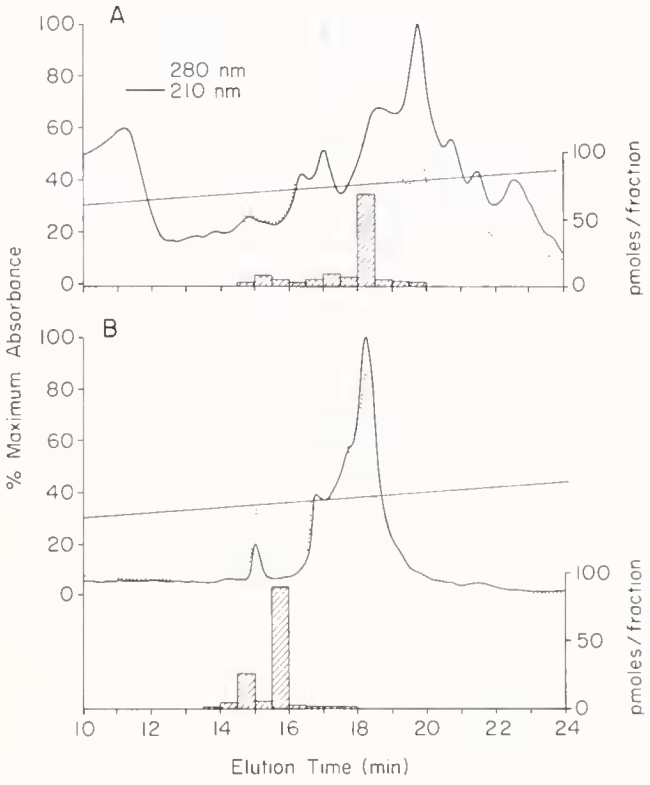

Figure 2. Further HPLC fractionation of the immunoreactive peak from Figure 1 on an Aquapore RP-300 column (220 × 2.1 mm). Loading, gradient and solvents as in Figure 1, but the flow rate is 0.5 ml/min. A. The immunoreactive peak from Figure 1 is diluted with 0.1% TFA and chromatographed. B. The immunoreactive peak from A is oxidized with hydrogen peroxide and chromatographed. A delay of about 0.2 ml (0.4 min) between the UV detector and fraction collector is not corrected for in the figure, so the peak of immunoreactivity corresponds to the UV peak at 15 min.

the elemental composition. The greater than predicted intensities of the higher mass ions (*e.g.*, 2343 and 2344) is probably due to a partial reduction of the disulfide bond during analysis (this would add 2 to the molecular ion).

Similarly, the signal at m/z of 2325 may correspond to a portion of the peptide in which the methionine sulfoxide was reduced back to methionine. Both of these reactions are promoted by the sample matrix, a mixture of dithiothreitol and dithioerythritol made strongly acidic with camphor sulfonic acid.

Taking the FABms data together with the sequence analysis, we conclude that the fish brain peptide has the sequence shown (Fig. 5).

The synthetic *Fundulus* brain peptide had the same elution time as the natural peptide, and the oxidized synthetic material had the same elution time as the oxidized natural peptide, when run under the same HPLC conditions as those used in the purifications. The *Fundulus* brain peptide eluted much later than the synthetic eel heart ANP and slightly later than rat ANP. The oxidized form of the synthetic *Fundulus* peptide has the same retention time as rat ANP.

Vasorelaxant activity

The synthetic *Fundulus* brain peptide is approximately equipotent to eel ANP in relaxing toadfish aortic rings, and both are very similar in potency to rat ANP (Fig. 6).

Immunoreactivity

In the RIA, using either eel ANP or rat ANP as trace, the *Fundulus* ANP peptide is about equiactive to rat ANP, and 3 to 5 fold more active than eel ANP (Fig. 7). All three of the peptides used to generate the curves shown in Figure 7 were quantified by amino acid analysis, so we are confident that the *Fundulus* and rat peptides are very similar in potency, and that both are slightly more potent than the eel peptide. The eel and *Fundulus* peptides consistently gave log/logit slopes of about −1, but the slope with the rat peptide was steeper.

Discussion

We have isolated and sequenced an ANP-like peptide from the brain of *Fundulus heteroclitus*. Like other ANPs and BNPs, this new natriuretic peptide contains a highly conserved, 17-residue disulfide bonded ring; regions outside the ring are, as usual, poorly conserved (Fig. 5). Within the ring, the killifish peptide is most sim-

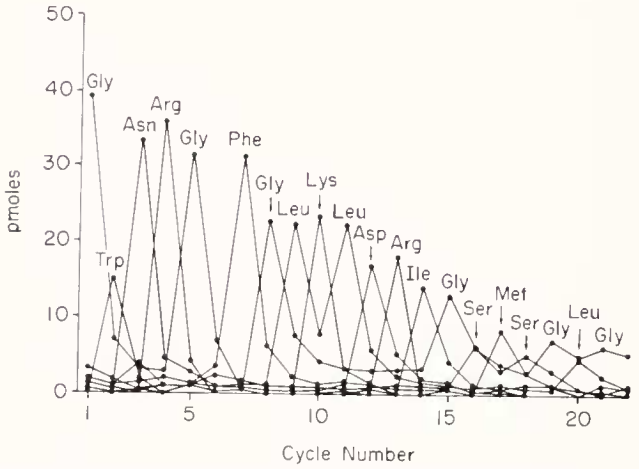

Figure 3. The yields of 11 PTH amino acids at each cycle of the sequencer analysis of the mildly oxidized peptide (Applied Biosystems): the value shown for serine is the sum of serine and dehydroalanine. The assignment for each position is shown. No assignment could be made for position 6, or for 22 and later. An increase in dehydroalanine at position 6 is consistent with this residue being a half cystine, but cystine cannot normally be identified without some pre-sequencing modification.

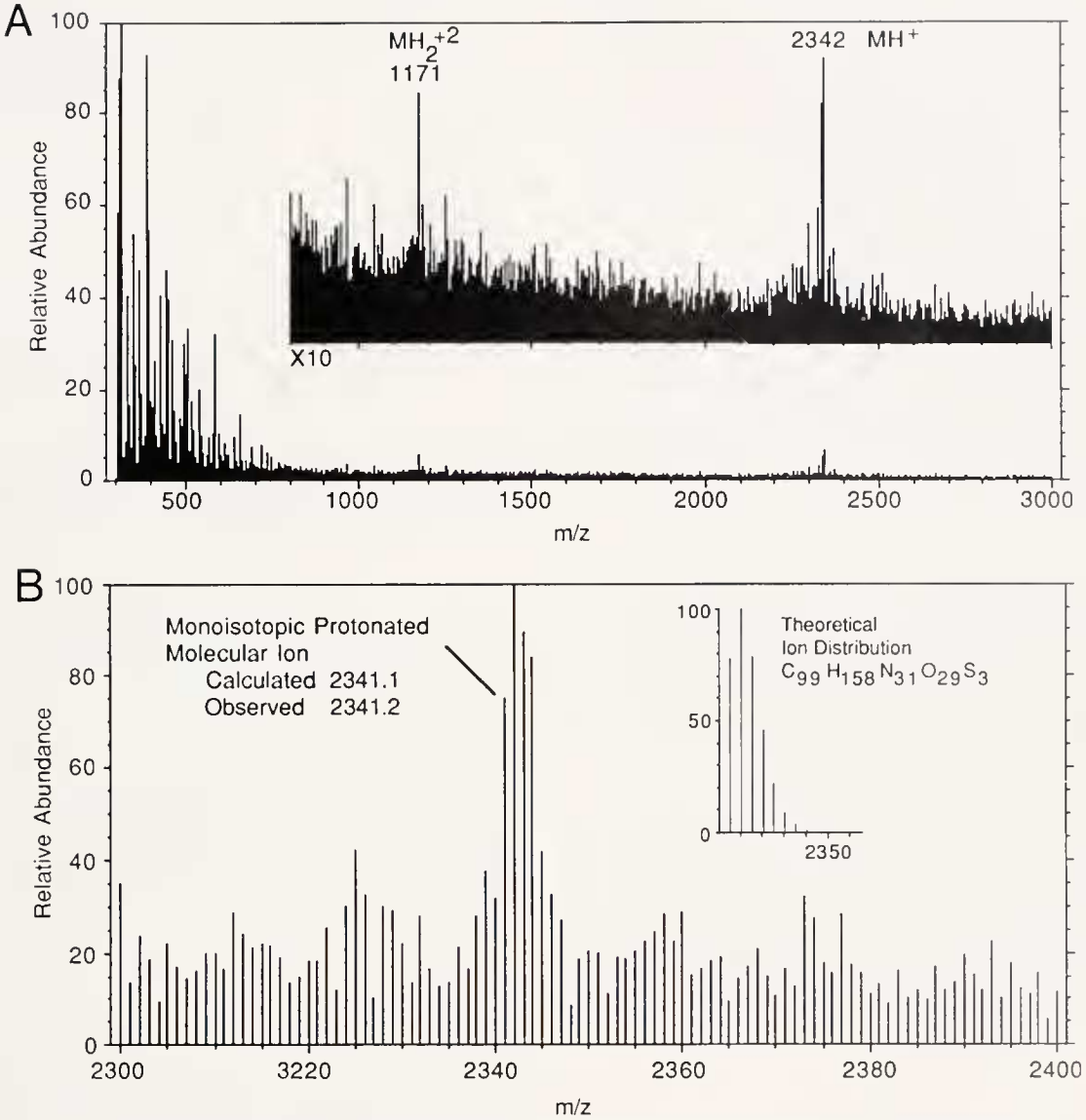

Figure 4. Positive ion FAB mass spectrum of the purified, mildly oxidized peak. A. Scan up to 3000 nominal mass. In the inset the x-axis is unchanged, but the y-axis is expanded 10-fold. The masses shown are rounded to the nearest integer. The two labeled ion clusters are the singly and doubly protonated molecular ions. B. The mass region around 2342, expanded to show the singly protonated molecular ion cluster. The theoretical ion distribution expected for a compound with the elemental composition found is shown in the inset.

ilar to an ANP isolated from eel heart (2 amino acid differences), and both fish peptides are quite similar to a peptide isolated from porcine brain (3 differences within the ring; Fig. 5).

The complete absence of a C-terminal "tail" is a unique feature not previously reported for any natriuretic peptide. The FABms data establish that the peptide sequenced had no tail, but we cannot completely rule out the possibility that a tail was lost by proteolysis during purification. Such degradation is unlikely, however, because the retention time of the immunoreactive peak remains the same when extracts are prepared in other ways (data not shown), and Y. Takei (pers. comm.) has sequenced an eel brain ANP-like peptide that also has no tail. Still, this question will not be settled until the cDNA encoding the precursor has been isolated.

The C-terminal tail seems to be irrelevant to either the relaxing activity of the peptide or its immunoreactivity. Thus, synthetic killifish peptide and eel ANP (which has a C-terminal tail) are equipotent in relaxing toadfish aor-

G W N R G C F G L K L D R I G S M S G L G C	Killifish brain
S K S S S P C F G G K L D R I G S Y S G L G C N S R K	eel heart
S P K T M R D S G C F G R R L D R I G S L S G L G C N V L R R Y	pig BNP
?S P K M M H K S G C F G R R L D R I G S L S G L G C N V L R K Y	dog BNP*
?S P K M V Q G S G C F G R K M D R I S S S S G L G C K V L R R H	human BNP*
M M R D S G C F G R R I D R I G S L S G M G C N G S R K N	chicken heart
-N S K M A H S S S C F G Q K I D R I G A V S R L G C D G L R L F	rat BNP*
-N S K M A H S S S C F G Q K I D R I G A V S R L G C D G L R Q F	rat isoANP
S L R R S S C F G G R M D R I G A Q S G L G C N S F R Y	human ANP
S L R R S S C F G G R I D R I G A Q S G L G C N S F R Y	rodent ANP
S S D C F G S R I D R I G A Q S G M G C G R R F	frog heart

Figure 5. The amino acid sequence of the *Fundulus* brain ANP-like peptide compared to other ANP peptides. The residues in these other peptides that are identical to those in *Fundulus* are boxed. The one-letter abbreviations for the amino acids are used. *Predicted from cDNA sequence. ?Exact length of predominant peptide is unknown. –These peptides are longer than shown.

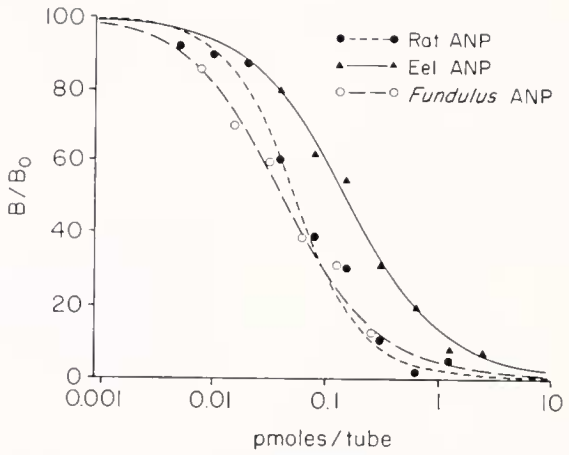

Figure 7. A comparison of the immunoreactivity of rat ANP (1–28), eel ANP, and *Fundulus* brain ANP-like peptide using an RIA with an antiserum to rat ANP and with iodinated eel ANP as trace.

tic rings (Fig. 6). Moreover, the new peptide is as immunoreactive as rat ANP in an RIA which employs the eel ANP as trace. The latter result is surprising since the RIA antiserum was raised to the rat peptide. Finally, we conclude that the apparent functional unimportance of the C-terminal tail is consistent with the dissimilarity of its sequence from one peptide to another (Fig. 5).

In mammals, the levels of ANP-like immunoreactivity in the heart are orders of magnitude higher than those of any other tissue, but such a tissue distribution is not a general characteristic of fish. For example, Galli *et al.* (1988) measured roughly equal levels of immunoreactivity in the brains and hearts of several species of teleosts using the same antiserum employed in our experiments.

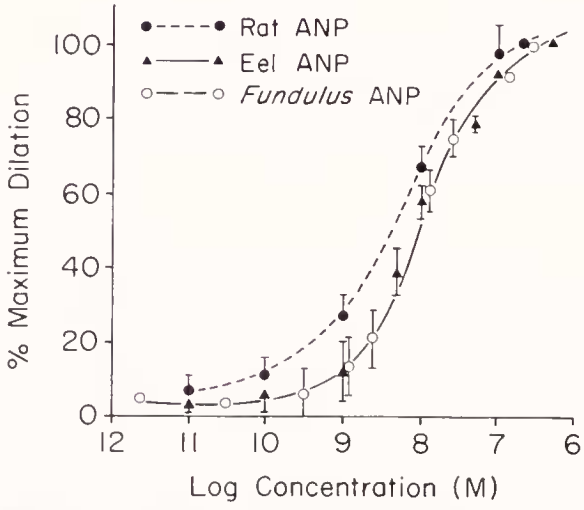

Figure 6. A comparison of the relaxing activity on rings of toadfish (*Opsanus beta*) ventral aorta, of rat ANP (1–28), eel ANP, and *Fundulus* brain ANP-like peptide.

Because this antiserum reacts about equally well with the *Fundulus* and eel peptides (Fig. 7), and because the eel peptide was isolated from heart, and the *Fundulus* from brain, the simplest interpretation of the data of Galli *et al.* (1988) is that heart and brain have roughly equal levels of peptide. Still, the immunoreactivity in the eel heart is (as in mammals) mostly in a high molecular weight form (Takei *et al.*, 1990), and this form may not be as immunoreactive as the smaller molecules that have been isolated and synthesized, and which we have used as standards.

Fish plasma contains ANP-like immunoreactivity, and the levels decrease with adaptation to reduced salinity (Galli *et al.*, 1988; Evans *et al.*, 1989). But neither the identity of the circulating peptide, nor its source, is known with certainty. In rats, even though the heart contains much more immunoreactivity than the brain, changes in hypothalamic and pituitary secretion of ANP markedly affect the blood levels of ANP (Baldissera *et al.*, 1989). Therefore, in species of teleosts like *Fundulus*, where levels of natriuretic peptide are about equal in brain and heart, the brain may be the major source of circulating peptide.

Acknowledgments

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